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Alternations in hepatic expression of fatty-acid metabolizing enzymes in ArKO mice and their reversal by the treatment with 17β-estradiol or a peroxisome proliferator☆

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Abstract

We generated aromatase gene knockout mice (ArKO mice) by targeting disruption of *Cyp19*, which encodes an enzyme responsible for conversion of androgens to estrogens. We found that ArKO males developed hepatic steatosis spontaneously with aging, indicating that the function of *Cyp19* is required to maintain constitutive lipid metabolism in male mice. Plasma lipoprotein analysis using a gel permeation chromatography revealed that high density lipoprotein (HDL)-cholesterol levels were slightly higher in ArKO males than in wild-type males, whereas no other obvious alternations in the profiles were detected. Nevertheless, analysis of lipoprotein compositions by SDS-polyacrylamide gel electrophoresis demonstrated apparent reduction in the amounts of apolipoprotein E, functioning in receptor-mediated clearance of lipoproteins in the liver, in the IDL/LDL fraction of ArKO males as compared with that of wild-type males. Biochemical analysis on the ArKO livers revealed suppression of mRNA expression and activity of enzymes involved in fatty acid β-oxidation. The impairment was reversed to the wild-type levels by treatment with 17β-estradiol or bezafibrate, the latter is a synthetic peroxisome proliferator. These findings indicated a pivotal role of estrogen in supporting constitutive hepatic expression of genes involved in fatty acid β-oxidation and in maintaining lipid homeostasis.

Keywords: Aromatase; *Cyp19*; Knockout mouse; Hepatic steatosis; Fatty acid β-oxidation; Peroxisome proliferator-activated receptor α

1. Introduction

Estrogens play important roles in the regulation of reproductive functions in both females and males. The steroids also have been implicated in the pathophysiology of a number of disease states including breast cancer, endometrial cancer, endometriosis, osteoporosis and cardiovascular disorders [1,2]. Hepatic production of apolipoprotein AI (apoAI), an apolipoprotein associated with high density-lipoprotein (HDL), and plasma HDL concentrations were shown to be increased on the treatment of postmenopausal women with estrogen [3]. Furthermore, in rats and rabbits, physiological doses of estrogen increased very low density-lipoprotein (VLDL) production, whereas higher doses of estrogen exert profound hypolipidemic effects [4], probably due to upregulation of lipoprotein clearance mediated by low density-lipoprotein (LDL)-receptors in the liver. Estrogens are also documented to exert anti-atherogenic effects in animal models, in part, through affecting both production and catabolism of lipoproteins [5,6]. Thus, elucidation of molecular mechanisms by which estrogens regulate production and catabolism of lipoproteins is an important issue for therapeutic application of estrogens [7].

Biosynthesis of estrogens is catalyzed by an enzyme complex consisting of two proteins, one is a specific form of cytochrome P450, termed aromatase cytochrome P450 (CYP19) and the other is a ubiquitous enzyme, NADPH-cytochrome P450 reductase [8]. Recently, we generated mice lacking aromatase activity (ArKO mice) by targeting disruption of *Cyp19* using homologous recombination techniques [9,10]. This ArKO mouse offers new opportunities to study physiological roles of estrogen in vivo including lipid metabolism.

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In the present study, we report consequences in plasma lipoprotein profiles caused by the *Cyp19* disruption and effects of 17β-estradiol (E_2)-supplementation on the profile. We also showed impairment in the expression of fatty acid-metabolizing enzymes in peroxisomes and mitochondria of the ArKO hepatic cells. The impairment is restored to the wild-type levels when ArKO males were treated with E_2 or bezafibrate, a synthetic ligand specific for peroxisome proliferator-activated receptor, PPAR-α. These findings clarify the fundamental role of estrogen in constitutive expression of fatty acid-metabolizing enzymes to maintain hepatic lipid homeostasis.

2. Materials and methods

2.1. Animal care and treatment

The aromatase cytochrome P450 gene (*Cyp19*) was disrupted by homologous recombination [9,10]. All animals were maintained on a 12 h light/dark cycle at 22–25 °C. They are fed with a standard rodent chow (NMF) (Oriental Yeast, Tokyo, Japan) and water ad libitum. ArKO male mice and their wild-type male siblings at 6 months of age were used in the present experiments. ArKO mice were divided into three groups: the first group of mice did not receive any treatment (control), the second group of mice received subcutaneous injections with 7.5 μg/mouse of 17β-estradiol (E_2) every fourth day for the first 3 weeks after birth and once a week with 0.75 μg E_2/mouse thereafter (ArKO + E_2), and the third group of ArKO mice received 0.5% of bezafibrate dietary at 6 months of age for 8 weeks (ArKO + Bez). Five mice were used for each group. Animal care and experiments were carried out in accordance with institutional animal care regulations.

2.2. Histology on the liver

Liver tissues were routinely fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and sectioned for hematoxylin- and eosin-staining. For electron microscopy, the tissues were cut in small pieces, immersed in a fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at 4 °C followed by postfixation in 1% osmic acid in 0.1 M cacodylate buffer (pH 7.2) for 1 h at 4 °C. After fixation, the tissues were rinsed, dehydrated in graded ethyl alcohol and embedded in a Spurr's epoxy resin. Ultra thin sections were doubly contrasted with uranyl acetic acid and lead citric acid, and observed in a Hitachi H-700H electron microscope.

2.3. Fatty acid β-oxidation activity

Fatty acid β-oxidation activity was measure as described previously [12]. (1-^{14}C)Tetracosanoic acid (C24:0), (1-^{14}C)palmitic acid (C16:0) and (1-^{14}C)lauric acid (C12:0) (55 mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO, USA) were used at 50 μM as substrates. After reaction was ended, radioactive degradation products in the water phase were counted. Fatty acid β-oxidation activity was expressed as nanomolars per minute per liver [10].

2.4. Analysis of mRNA expression for enzymes involved in fatty acid β-oxidation

Expression of genes involved in fatty acid β-oxidation was analyzed by Northern blotting [11]. Total liver RNA was obtained from fresh livers using the acid guanidinium thiocyanate-phenol-chloroform extraction method. RNA was separated on 1% agarose gels containing formaldehyde and transferred to nylon membranes. The membranes were incubated with ^{32}P-labeled cDNA probes. The cDNAs used were catalase, very long fatty acyl-CoA synthetase (VLACS), peroxisomal acyl-CoA oxidase (AOX), medium-chain acyl-CoA dehydrogenase (MCAD), cytochrome P450 4A1 (CYP4AI), and GAPDH. Quantification of the bands were done by BAS 2000 (Fuji Photo Film, Tokyo, Japan).

2.5. Lipoprotein profiles and sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis

Blood samples were collected from the tail vein of mice at 6–8 months of age, that had been fasted for 5 h. EDTA at a final concentration of 10 mM was used as an anticoagulant [13]. Plasma was obtained by centrifugation of the blood at 14,000 rpm (microcentrifuge) for 10 min at 4 °C and pooled (100 μl/mouse, from five mice). Fifty microliter of the plasma mixture was subjected to gel permeation chromatography using a Superose 6 column equipped with a SMART system (Amarsham Pharmacia Biotech). The column was equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl and 1 mM EDTA [14]. The samples were eluted at a flow rate of 40 μl/min and fractions (80 μl) were collected. A 20 μl aliquot of each fraction was used to measure concentrations of cholesterol and triglyceride by colorimetric methods using kits (Nissui, Tokyo Japan). For analysis of protein compositions associated with each lipoprotein, plasma samples were subjected to separation by ultracentrifugation as described by Havel et al. [15]. A 3 μl aliquot of the HDL fraction and 50 μl aliquots of VLDL and LDL fractions, which were precipitated with trichloroacetic acid and rinsed with acetone before analysis, were used for apolipoprotein analysis on 4–15% SDS-polyacrylamide gels. Proteins were stained with Coomassie blue. Bands corresponding to apoAI and apoE in the gels were identified by Western blot analyses using antibodies raised against human apoAI (Roche Diagnostics, Tokyo) and murine apoE (Cortex Biochem, San Leandro), respectively. Bands corresponding to apoB-100 and apoB-48 were estimated based

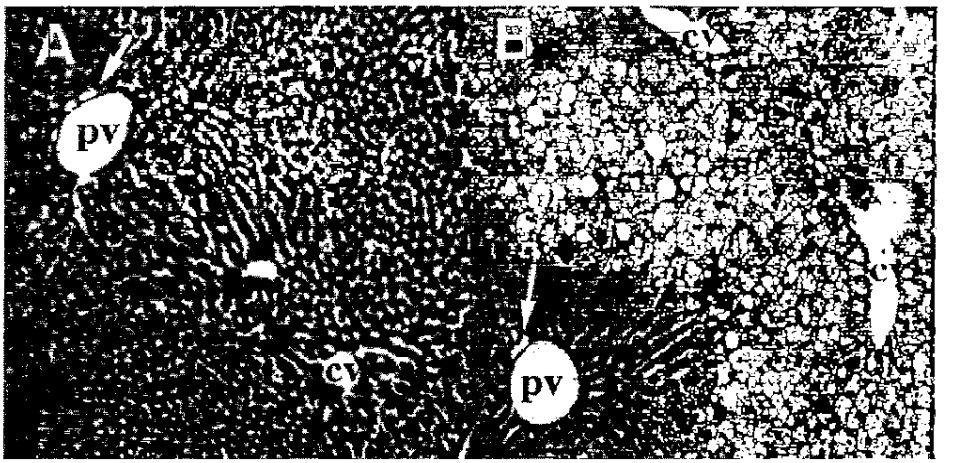

Fig. 1. Light micrographs of the livers from wild-type (A) and ArKO mice (B) at 8 months of age. Liver lobules in ArKO mice (B) are composed of hepatocytes showing marked accumulation of micro- and macrovesicular lipid droplets, which are rimmed with narrower periportal zone consisted of cells appearing normal. Portal vein and central vein are indicated by pv and cv, respectively. Arrow indicates hepatic artery. Scale bar; 500 μm. Hematoxylin- and eosin-staining.

on the migration-profile on the SDS-polyacrylamide gel in the LDL-rich fraction [16].

2.6. Statistic analysis

Data were analyzed using Student *t*-test or Wilcoxon signed rank test.

3. Results

3.1. Accumulation of lipid droplets in hepatocytes in ArKO males

ArKO male mice are indistinguishable externally from the wild type littermates except that they become bigger due to accumulation of fat in the gonadal fat pads with aging. Histological examination of the liver by light microscopy revealed micro- and macro-vesicular steatosis in hepatic cells at centrilobular and intermediate zones in the lobules (Fig. 1A and B). However, hepatocytes at the periportal zone remained intact. Such zonation in steatosis was always clearly observed within the liver lobules. At the ultrastructural level, hepatic cells from ArKO males exhibited presence of numerous lipid droplets in the cytosol, while other organelle such as mitochondria appeared to be normal (Fig. 2).

3.2. Plasma lipoprotein analysis

We employed a gel permeation chromatography using a Superose 6 column to analyze lipoprotein profiles in ArKO males. As shown in Fig. 3, no obvious alternations in the profiles were noticed, except that HDL-cholesterol levels were slightly higher in ArKO males than those in wild-type males. Compositional differences in apolipoproteins were analyzed by SDS-polyacrylamide gel electrophoresis using VLDL, IDL/LDL and HDL fractions prepared by ultracentrifugation. Reduction in the amounts of apolipoprotein E and possibly apolipoprotein B were observed in the IDL/LDL fraction of ArKO males as compared with that of wild-type males (Fig. 4).

3.3. Suppression of fatty acid β-oxidation activity in the liver of ArKO males

The basal levels of total fatty acid β-oxidation activity were measured using three types of substrates different in length of the carbon chain. Lauric acid (C12:0) and palmitic acid (C16:0) are metabilized preferentially by mitochondrial enzymes and tetracosanoic acid (C24:0) is metabolized by the enzymes including those localized in peroxisomes. As seen in Fig. 5, the activities in ArKO males were obviously lower than those of wild-type males with either substrate, C12:0 (13.4 ± 1.7 versus 3.9 ± 0.1 nmol/min per liver; $P < 0.001$), C16:0 (66.0 ± 3.5 versus 19.8 ± 2.9 nmol/min per liver; $P < 0.001$) and C24:0 (3.26 ± 0.15 versus 0.73 ± 0.08 nmol/min per liver; $P < 0.001$). In contrast, the basal constitutive activity of total fatty acid β-oxidation in ArKO females was much higher than that of ArKO males. Especially, comparable levels of activity to the wild-type liver were observed when fatty acids with longer carbon chain were used as a substrate, C16:0 (62.7 ± 3.7 versus 57.1 ± 3.2 nmol/min per liver) and C24:0 (2.76 ± 0.24 versus 2.89 ± 0.22 nmol/min per liver).

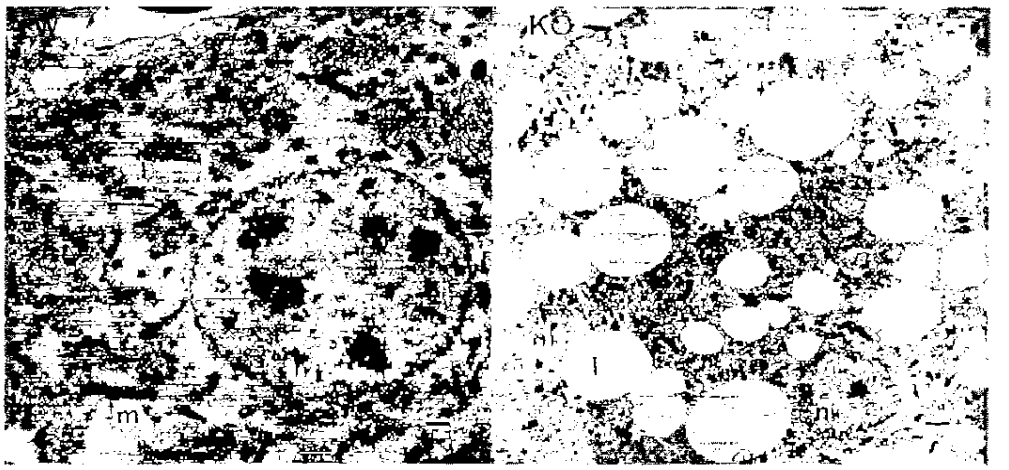

Fig. 2. Electron micrographs of liver from wild-type and ArKO male mice at 8 months of age. Thin sections of the liver were prepared, stained with lead and uranium salts, and viewed at a magnification of 10,000×. (W) wild-type liver, (KO) ArKO liver, (l), (m) and (n) indicate lipid droplet, mitochondrion and nucleus, respectively.

Expression levels of mRNAs for hepatic peroxisomal, mitochondrial, and microsomal enzymes involved in fatty acid oxidation were analyzed by Northern blot hybridization. As shown in Fig. 6, constitutive mRNA expression of mitochondrial MCAD and of three peroxisome-associated enzymes, VLACS, AOX, and catalase, were significantly lowered in ArKO males than that in wild-type males, whereas microsomal CYP4A1 mRNA expression, which is well known to be induced by PPAR-α stimulation [17], was significantly enhanced in ArKO males. These findings indicate that the PPAR-α signaling pathways is functional and might be activated in the ArKO liver, although the expressions of peroxisomal and mitochondrial enzymes are suppressed.

3.4. Effect of E_2 and bezafibrate on the liver of ArKO males

Lipoprotein profiles of plasma from ArKO males supplemented with E_2 was analyzed by gel permeation chromatography to examine the effect of E_2 on the liver of ArKO males. Upon the treatment, the levels of triglyceride in the VLDL fractions and of cholesterol in the IDL/LDL fractions were increased (Fig. 3C). Biochemical analysis demonstrated that the treatment resulted in reversal of mRNA levels for enzymes involved in fatty acid β-oxidation nearly to the levels of the wild-type males (Fig. 7). In addition, when the mutant animals were treated for 2 months with bezafibrate, a synthetic ligand for PPAR-α,

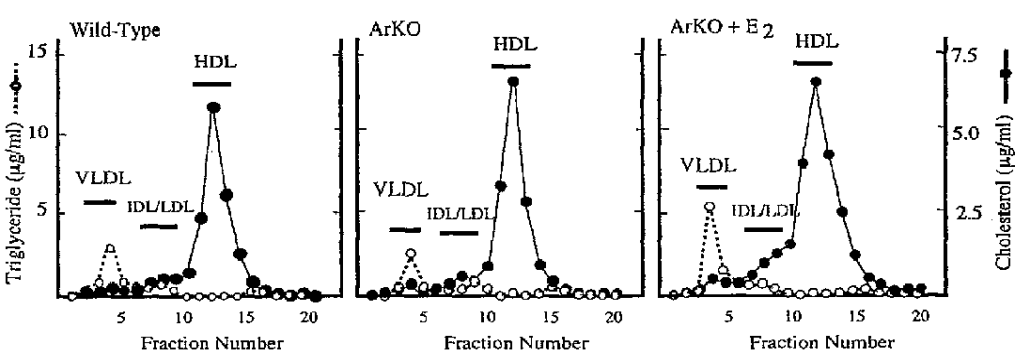

Fig. 3. Lipoprotein profiles of plasma. Wild-type male mice, ArKO male mice or ArKO male mice supplemented with E_2 were fasted for 5 h before collecting plasma. Plasma from mice of each group were pooled and subjected to gel permeation chromatography using a Superose 6 column. Triglyceride (open circle) and cholesterol (closed circle) contents of each fraction were measured as described in materials and methods. The fractions rich in VLDL, IDL/LDL and HDL were indicated.

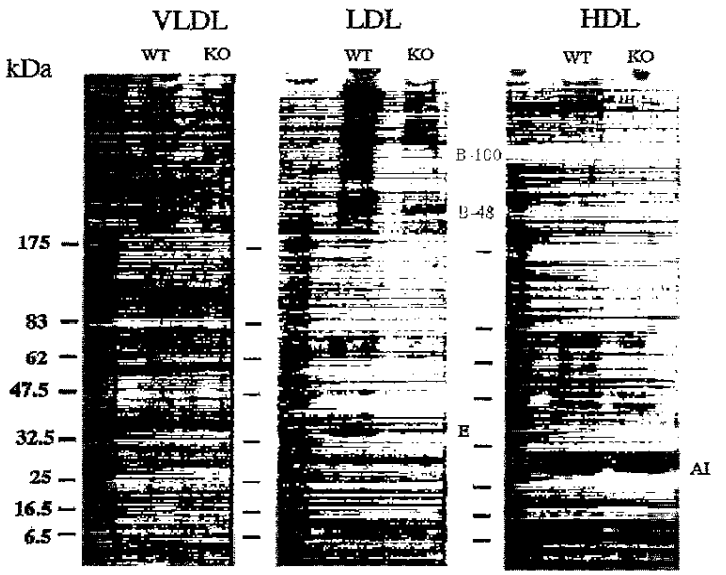

Fig. 4. SDS-gel electrophoresis of lipoprotein fractions from wild-type and ArKO mice. The apolipoproteins in VLDL, IDL/LDL and HDL fractions prepared by ultracentrifugation were subjected to electrophoresis on 4–15% SDS-polyacrylamide gels. Proteins were stained with Coomassie blue. The positions of apoB-100, apoB-48, apoE and apoAI are indicated.

expression of mRNA involved in the β-oxidation are markedly increased (Fig. 7). However, the expression level of a microsomal enzyme, CYP4A1, is not reduced to the level of wild-type mice, confirming that *Cyp4A1* is a gene of which transcription is induced by activated PPAR-α. Histological examination of the liver treated with bezafibrate demonstrates restoration of the hepatic steatosis (data not shown).

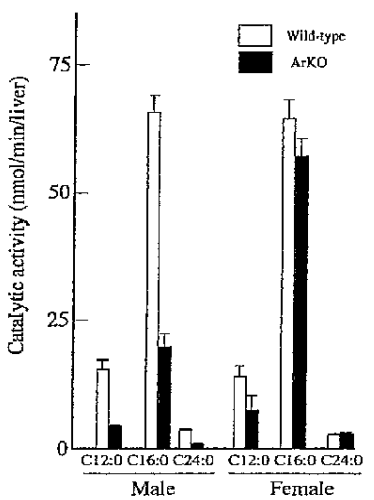

Fig. 5. Impairment in hepatic fatty acid β-oxidation activity of ArKO mice. Peroxisomal β-oxidation activity was assessed using tetracosanoic acid (C24:0) as a substrate, and mitochondrial β-oxidation activity was measured using palmitic acid (C16:0) and lauric acid (C12:0). The bars represent the mean ± S.D. from at least six samples in each genotype. The differences between wild-type and ArKO male mice were statistically significant ($P < 0.001$).

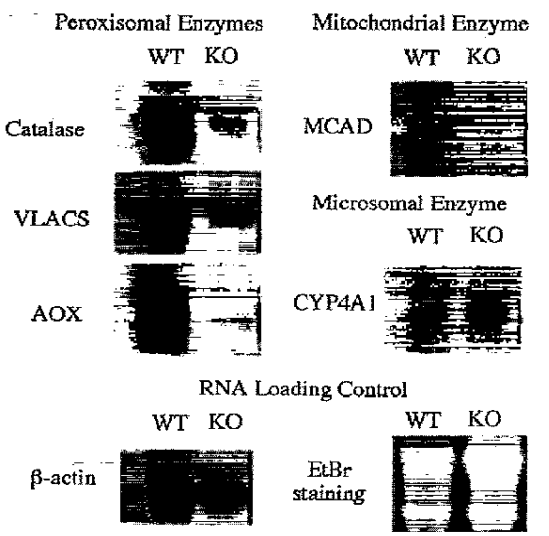

Fig. 6. Northern blot analysis of gene expression in ArKO mice. Total cellular RNA was prepared from the livers of wild-type and ArKO mice. Probes of peroxisomal and mitochondrial enzymes essential for β-oxidation were used in the analyses. Equal amount (15 μg) of total RNA was applied in each lane.

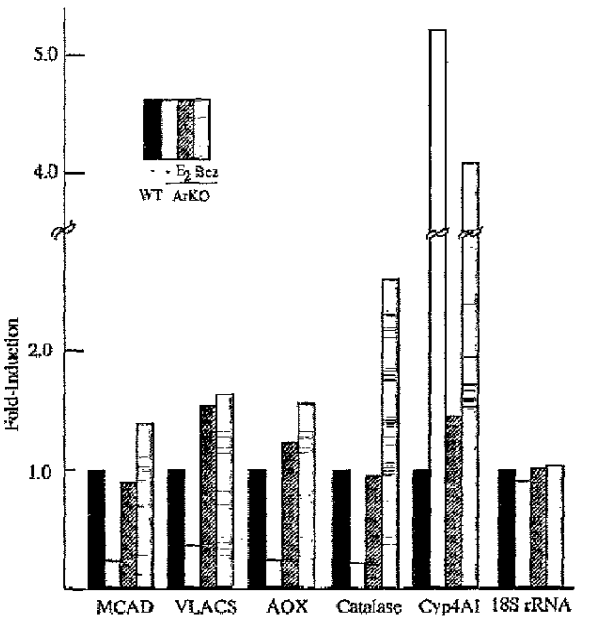

Fig. 7. Amelioration of suppressed levels of expression of mRNAs for fatty acid β-oxidation in the liver. Expression levels of mRNA coding for enzymes involved in fatty acid oxidation were analyzed by Northern blot hybridization using total RNA isolated from the livers of wild-type males (closed bar), untreated ArKO males (open bar), ArKO males treated with E_2 (slashed bar) and ArKO males treated with bezafibrate (dotted bar). Equal amount (15 μg) of total RNA was separated in 1% formaldehyde/agarose gels. Probes used for the analyses were medium-chain acyl-CoA dehydrogenase (MCAD) for mitochondrial enzyme, very long fatty acyl-CoA synthetase (VLACS), acyl-CoA oxidase (AOX), and catalase for peroxisomal enzymes, and cytochrome P450 4A1 (CYP4A1) for microsomal enzyme. The amount of 18S rRNA was used as a loading control of RNA. Quantification of the bands were done by BAS 2000.

4. Discussion

We presented here that the disruption of *Cyp19* resulted in spontaneous development of massive hepatic steatosis in male mice. While plasma lipoprotein profiles on gel permeation chromatography did not exhibit obvious differences between wild-type and ArKO mice, apolipoproteins associated with the IDL/LDL fraction was apparently different showing reduction in the amounts of apoE and apoB in ArKO males. Furthermore biochemical analysis of the ArKO liver revealed marked suppression of mRNA expression of enzymes involved in peroxisomal and mitochondrial β-oxidation in ArKO mice as compared with that of wild-type males. Consistently, we observed reduction in the β-oxidation activities in ArKO males. Therefore, these observations suggest that estrogen plays important roles in vivo in lipid metabolism. In consistent, study on the mice lacking one of the estrogen receptor genes, ERα, demonstrated enhanced accumulation of fat in adipocytes [18]. Furthermore, it was reported that tamoxifen, a potent antagonist of estrogens, frequently induces hepatic steatosis without significant changes in body mass index among non-alcoholic, non-diabetic, and non-obese women with breast cancer [19–21]. These clinical observations in humans also suggest importance of the estrogen receptor-mediated signaling pathway in basal hepatic lipid homeostasis.

We observed that upon supplementation with E_2, ArKO males show normal expression patterns of mRNAs coding for hepatic enzymes involved in peroxisomal and mitochondrial β-oxidation. The supplementation also prevents ArKO mice from development of hepatic steatosis. Analysis of plasma lipoprotein profiles demonstrated that on the supplementation, a significant increase in the amounts of VLDL and IDL/LDL was observed, as judged by the contents of triglycerides and cholesterols, respectively. These effects of estrogen on the plasma lipoprotein profiles were reported using other mouse strains [22,23], in which estrogen was shown to increase synthesis and secretion of apoB, a major apolipoprotein associated with VLDL and LDL, but not the amounts of hepatic LDL receptor proteins [23]. Thus, taken these observation together, we assume that supplementation with estrogen causes enhancement in fatty acid β-oxidation activity in the liver and that the supplementation also causes increases in production and secretion of VLDL into the plasma from the liver, which consequently prevents the liver from unusual accumulation of fatty acids. Nevertheless, it is not clear whether or not estrogens directly control the hepatic functions by regulating transcription of genes expressed in the liver.

PPAR-α is a well established regulatory molecule in lipid homeostasis in the liver. It acts as a physiological sensor of lipid levels and also as a transcriptional regulator of an array of lipid metabolism-related genes [24–26]. In the present study, we showed that dietary administration of bezafibrate, an activator of the PPAR-α signaling-pathway, to ArKO males for 2 months resulted in amelioration of severe hepatic steatosis. These results imply that the PPAR-α signaling-pathway is not functional enough in the liver of ArKO males. Male mice lacking the functional PPAR-α gene show reduction in constitutive mitochondrial β-oxidation activity and development of centrilobular steatosis in the liver [27,28], both of which are phenotypes observed in ArKO males. Thus, although the expression of *Cyp4A1*, which is one of the genes known to be induced by activated PPAR-α [17], is enhanced in the ArKO liver, impairment in the PPAR-α signaling-pathways might not be unlikely in ArKO mice.

The presence of sexual dimorphism in the hepatic lipid metabolism is implicated by the study on PPAR-α-deficient mice in which estrogen signaling pathway is highlited to be important [24]. However, our present study demonstrates that the gender-related difference in the fatty acid β-oxidation activity exists in ArKO mice. These findings indicate that estrogen is involved in, but not essential for the establishment of the gender-related lipid metabolism.

In conclusion, our present study demonstrates that estrogen plays obviously important roles in hepatic lipid

metabolism in mice as well as humans. Furthermore, ArKO mice provide us with a great opportunity to unveil regulatory mechanisms, by which gender-related lipid metabolism is established.

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